Chapter 3

Insulin induces a hypercontractile airway smooth muscle phenotype.

Reinoud Gosens, S. Adriaan Nelemans, Maartje Hiemstra, Mechteld M. Grootte Bromhaar, Herman Meurs & Johan Zaagsma

European Journal of Pharmacology (2003) 481:125-131.

Abstract
This study aims to investigate the effects of insulin on bovine tracheal smooth muscle (BTSM) phenotype *in vitro*. Contractility of muscle strips and DNA-synthesis ([^{3}H]thymidine incorporation) of isolated cells were used as parameters for smooth muscle phenotyping. Insulin (1 μM) was mitogenic for BTSM and potentiated DNA-synthesis induced by other growth factors. In contrast, after pretreatment of unpassaged BTSM cells in culture, the mitogenic response induced by growth factors was strongly diminished, with no difference in the basal incorporation. Pretreatment of BTSM strips in organ culture with insulin increased maximal contraction to methacholine and KCl. These results show that insulin acutely augments DNA-synthesis in the presence of other growth factors. In contrast, insulin pretreatment induces a hypercontractile phenotype with a decreased mitogenic capacity. This mechanism may be involved in the putative negative association between asthma and type I diabetes. In addition, these findings may have implications for the use of aerosolized insulin in diabetes mellitus.

Introduction
Cultured airway smooth muscle (ASM) cells are known to develop a less contractile phenotype when exposed to serum-rich culture media and growth factors, characterized by a decreased shortening capacity and contractile protein expression, while the proliferative and synthetic capabilities of these cells are enhanced [1-3]. Phenotype switching is known to be regulated by extracellular matrix proteins that either promote (e.g. collagen type I, fibronectin) or inhibit (e.g. laminin) progression toward the less contractile and more proliferative state [4]. In Chapter 2, we have demonstrated that intact ASM, embedded in its own extracellular matrix, is also sensitive to phenotype changes induced by exogenously applied growth factors [5]. Progression to the less contractile state can be induced by serum, platelet-derived growth factor (PDGF), insulin-like growth factor-1 (IGF-1) and epidermal growth factor (EGF), which is linearly related to the mitogenic response of the growth factor applied [5].

It is unknown whether the observed relationship between proliferation and phenotypic modulation is shared by all growth factors that stimulate receptors with intrinsic tyrosine kinase activity, including insulin. Insulin is known to be mitogenic for cultured human ASM cells and to potentiate ASM mitogenesis induced by other receptor tyrosine kinase agonists, such as EGF, and by G-protein coupled receptor agonists, such as thrombin [6]. Consequently, one would expect insulin to promote progression toward the less contractile state. However, induction of functionally hypercontractile myocytes has been reported after treatment with serum-free media containing insulin [3]. Hence, it is of great interest to solve this discrepancy. Moreover, insight in the long-term effects of insulin on ASM phenotype is warranted in view of recent publications on the application of aerosolized insulin in diabetes mellitus [7-9]. Inducing a phenotype switch by this mode of administration could limit its use, especially in patients suffering from airway diseases. Moreover, long-term

effects of insulin could also explain the repeatedly reported negative association between type I diabetes and asthma [10;11].

Therefore, we investigated the effects of insulin on bovine tracheal smooth muscle (BTSM) phenotype in vitro, using both intact tissue and isolated cells, in which we measured contractility and proliferative responsiveness, respectively, as parameters for smooth muscle phenotype. Insulin was acutely mitogenic for BTSM cells and synergistically potentiated mitogenesis induced by PDGF, IGF-1 and EGF. However, *pre*-treatment with insulin induced a hypercontractile and hypoproliferative phenotype of these cells.

Methods

Tissue preparation and organ culture procedure

Bovine tracheae were obtained from local slaughterhouses and rapidly transported to the laboratory in Krebs-Henseleit (KH) buffer of the following composition (mM): NaCl 117.5, KCl 5.60, $MgSO_4$ 1.18, $CaCl_2$ 2.50, NaH_2PO_4 1.28, $NaHCO_3$ 25.00 and glucose 5.50, pregassed with 5 % CO_2 and 95% O_2; pH 7.4. After dissection of the smooth muscle layer and careful removal of mucosa and connective tissue, tracheal smooth muscle strips were prepared while incubated in gassed KH-buffer at room temperature. Care was taken to cut tissue strips with macroscopically identical length (1 cm) and width (2 mm). Tissue strips were washed once in sterile Dulbecco's modification of Eagle's medium (DMEM), supplemented with $NaHCO_3$ (7 mM), HEPES (10 mM), sodium pyruvate (1 mM), nonessential amino acid mixture (1:100), gentamicin (45 µg/ml), penicillin (100 U/ml), streptomycin (100 µg/ml) and amphotericin B (1.5 µg/ml). Next, tissue strips were transferred into suspension culture flasks and a volume of 7.5 ml medium was added per tissue strip. Strips were maintained in culture in an incubator shaker (37 ˚C, 55 rpm) for 8 days, refreshing the medium on day 4. Either fetal bovine serum (FBS) or insulin were present during the entire incubation period, when applied.

Isometric tension measurements.

Tissue strips, collected from suspension culture flasks, were washed with several volumes of KH-buffer pregassed with 5 % CO_2 and 95 % O_2, pH 7.4 at 37 ˚C. Subsequently, strips were mounted for isometric recording (Grass force-displacement transducer FT03) in 20 ml water-jacked organ baths, containing KH-buffer at 37 ˚C, continuously gassed with 5 % CO_2 and 95 % O_2, pH 7.4. During a 90 min equilibration period, with washouts every 30 min, resting tension was gradually adjusted to 3 g. In separate experiments it was established that strips stretched to 3 g passive tension responded optimally. Subsequently, muscle strips were precontracted with 20 mM and 30 mM isotonic KCl solutions. Following two wash-outs, basal smooth muscle tone was established by the addition of 0.1 µM (-)-isoprenaline and tension was re-adjusted to 3 g, immediately followed by two changes of fresh KH-buffer. Following another equilibration period of 30 min, cumulative concentration response curves were constructed to stepwise increasing concentrations of isotonic KCl (5.6 – 50 mM) or methacholine (1 nM – 100 µM). The

increase in KCl concentration was compensated for by substitution with NaCl to maintain iso-osmolarity. When maximal KCl or methacholine-induced tension was obtained, the strips were washed several times and basal tone was re-established using (-)-isoprenaline (10 μM).

Isolation of bovine tracheal smooth muscle cells
Tracheal smooth muscle was chopped using a McIlwain tissue chopper, three times at a setting of 500 μm and three times at a setting of 100 μm. Tissue particles were washed two times with the medium mentioned above, supplemented with 0.5 % FBS. Enzymatic digestion was performed in the same medium, supplemented with collagenase P (0.75 mg/ml), papain (1 mg/ml) and soybean trypsin inhibitor (1 mg/ml). During digestion, the suspension was incubated in an incubator shaker (Innova 4000) at 37 ˚C, 55 rpm for 20 min, followed by a 10 min period of shaking at 70 rpm. After filtration of the obtained suspension over 50 μm gauze, cells were washed three times in medium supplemented with 10 % FBS.

[^{3}H]Thymidine-incorporation
BTSM cells were plated in 24 well cluster plates at a density of 30,000 cells per well in 10 % FBS containing medium at 37 ˚C in a humidified 5 % CO_2-incubator. After attachment overnight, cells were washed two times with sterile phosphate buffered saline (PBS, composition (mM) NaCl, 140.0; KCl, 2.6; KH_2PO_4, 1.4; $Na_2HPO_4.2H_2O$, 8.1; pH 7.4). Subsequently cells were made quiescent by incubation for 72 h in serum-free medium supplemented with 0.1 % FBS, apo-transferrin (5 μg/ml) and ascorbate (100 μM). When pretreatment effects of insulin were studied, 0.1 % FBS was replaced for insulin (1 μM).

After quiescence, cells were washed with PBS and stimulated with mitogens in serum-free medium for 28 h, the last 24 h in the presence of [^{3}H]thymidine (0.25 μCi/ml), followed by two washes with PBS at room temperature and one with ice-cold 5 % trichloroacetic acid. Cells were treated with this trichloroacetic acid-solution on ice for 30 min; subsequently the acid-insoluble fraction was dissolved in 1 ml NaOH (1 M). Incorporated [^{3}H]thymidine was quantified by liquid-scintillation counting.

Data analysis
All data represent means ± s.e.mean from *n* separate experiments and EC_{50} was expressed as the concentration required to induce half the maximal effect (E_{max}). pD_2 values were calculated as $-\log EC_{50}$. The statistical significance of differences between data was determined by the Student's *t*-test for paired observations (two-tailed). Differences were considered to be statistically significant when $P < 0.05$.

Materials
DMEM and methacholine chloride were obtained from ICN Biomedicals (Costa Mesa, CA, U.S.A.). Fetal bovine serum, $NaHCO_3$ solution (7.5 %), HEPES solution (1 M), sodium pyruvate solution (100 mM), non-essential amino acid mixture, gentamycin

solution (10 mg/ml), penicillin/streptomycin solution (5000 U/ml / 5000 µg/ml), amphotericin B solution (250 µg/ml) (Fungizone) and trypsin were obtained from Gibco BRL Life Technologies (Paisley, U.K.). EGF (human recombinant), IGF-1 (human recombinant), PDGF (human recombinant), insulin (from bovine pancreas), apotransferrin (human) and soybean trypsin inhibitor were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A). [*Methyl*-^{3}H]thymidine (specific activity 25 Ci/mmol) was obtained from Amersham (Buckinghamshire, U.K.). Papain and collagenase P were from Roche Diagnostics (Mannheim, Germany). All other chemicals were of analytical grade.

Results

Acute effects of insulin on bovine tracheal smooth muscle DNA-synthesis

Acute effects of insulin on DNA-synthesis were assessed, using cells that were made quiescent in serum-free medium for a period of 3 days. Insulin (1 µM) increased [^{3}H]thymidine incorporation to 159 ± 11 % of basal (Figure 3.1A, P <0.01). In combination with other growth factors (PDGF, IGF-1, EGF), insulin induced a synergistic enhancement of the responses. Synergism was expressed as the difference between the sum of individual responses and the measured combined response. Interestingly, as compared to IGF-1 (10 ng/ml), PDGF (10 ng/ml)-induced and EGF (10 ng/ml)-induced incorporation were potentiated to a larger extent (Figure 3.1).

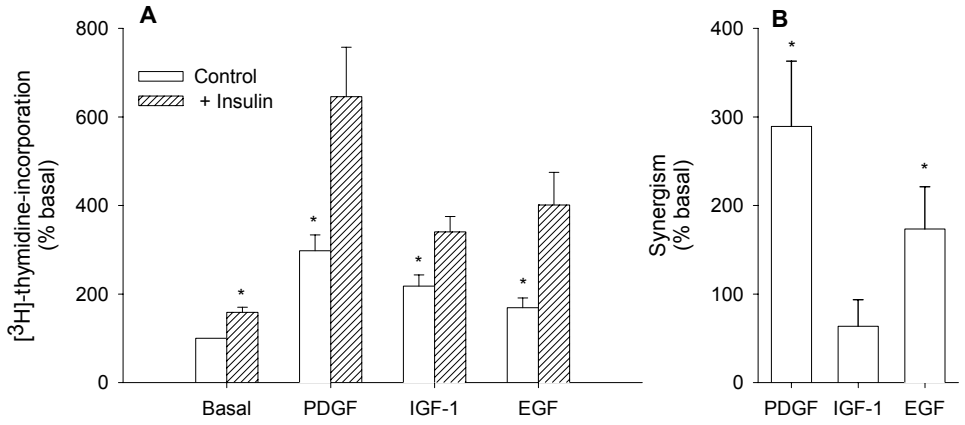

Figure 3.1 *A: [^{3}H]Thymidine incorporation of unpassaged BTSM cells. Basal responses and those in response to EGF (10 ng/ml), IGF-1 (10 ng/ml) and PDGF (10 ng/ml) were measured, both in the absence and presence of insulin (1 µM). B: Calculated synergism of DNA-synthesis of the applied growth factors due to the presence of insulin. Data represent means ± s.e.mean. of 4-5 experiments each performed in triplicate. * P < 0.05 compared to control basal; † P < 0.05 compared to absence of insulin.*

Effects of pretreatment with insulin on bovine tracheal smooth muscle DNA-synthesis

In order to investigate the effect of pretreatment with insulin on BTSM DNA-synthesis, cells were made quiescent in media with and without insulin (1 µM) for a period of 3 days. After this period, cells were washed and stimulated with growth factors. No difference in basal [^{3}H]thymidine incorporation could be observed for pretreatment without and with insulin that averaged 3,661 ± 803 and 3,459 ± 740 dpm/well (n=15), respectively. However, the mitogenic effect induced by PDGF (10 ng/ml), was significantly reduced after pretreatment with insulin. Similarly, a reduction in incorporated [^{3}H]thymidine was observed for IGF-1 (10 ng/ml), whereas the response to EGF (10 ng/ml) was suppressed completely (Figure 3.2). Analysis of the concentration-response relationship for PDGF showed that the observed decrease manifested itself both as a decrease in maximal effect and as a rightward shift, indicating a decreased sensitivity (E_{max} = 338 ± 26 and 207 ± 20 % of basal (*P*<0.001) and EC_{50} = 2.1 ± 0.7 and 4.0 ± 1.4 ng/ml (*P*<0.05) for pretreatment in medium without and with insulin, respectively, Figure 3.3).

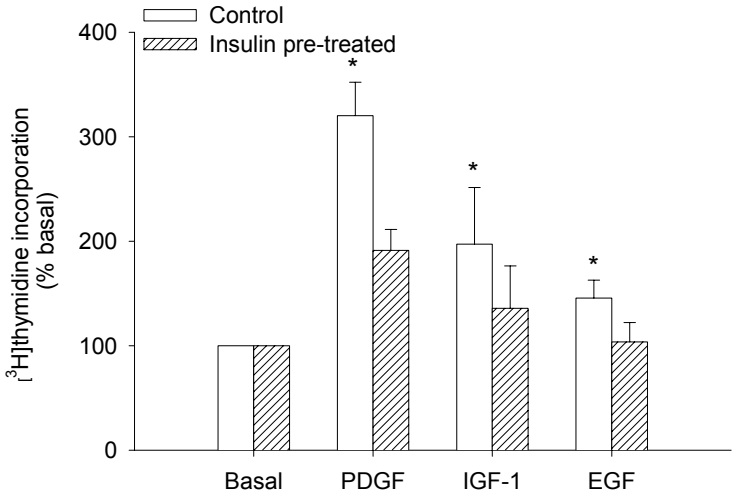

Figure 3.2 *[^{3}H]Thymidine incorporation of unpassaged BTSM cells, pre-treated with serum-free medium with or without insulin (1 µM) for a period of 3 days. Basal responses and those in response to EGF (10 ng/ml), IGF-1 (10 ng/ml) and PDGF (10 ng/ml) were measured. Data represent means ± s.e.mean. of 6 experiments each performed in triplicate. * P < 0.05 compared to basal; † P < 0.05 compared to serum-free pre-treatment.*

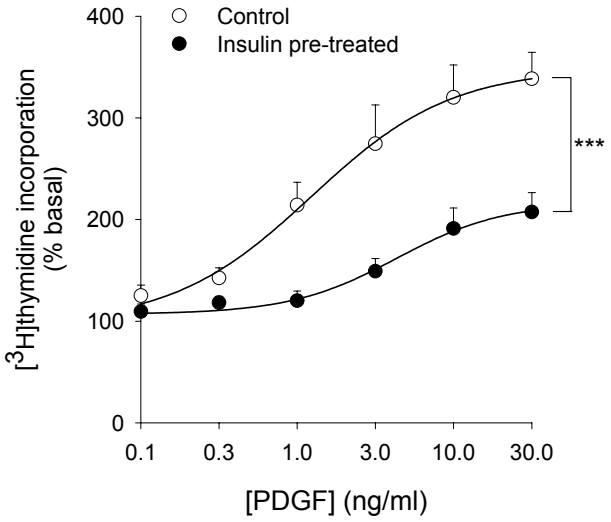

Figure 3.3 *PDGF-induced [^{3}H]thymidine incorporation of unpassaged BTSM cells, pre-treated with serum-free medium with or without insulin (1 μM) for a period of 3 days. Data represent means ± s.e.mean. of 6 experiments each performed in triplicate. *** P < 0.001.*

Effects of pretreatment with insulin on bovine tracheal smooth muscle contractility

The effects of insulin (1 μM) on BTSM phenotype were investigated using intact organ-cultured smooth muscle strips as described in Chapter 2. In view of the time course of the phenotypic switch in intact tissue (t½ = 2.8 days), strips were pretreated with insulin for a period of 8 days. As positive controls, some preparations were treated with 10 % FBS, known to switch to a less contractile phenotype. As expected, strips treated with 10 % FBS responded with a decrease in E_{max} for methacholine. No change in sensitivity (pD_2) was observed after treatment with 10 % FBS (Figure 3.4). In contrast, strips treated with insulin responded with an increase in maximal contraction for methacholine when compared to serum-free medium pre-treated strips. This increase was quantitatively similar to the decrease in E_{max} induced by 10 % FBS. In addition, a small but significant leftward shift could be observed in the dose-response relationship for methacholine after pretreatment with insulin (pD_2 = 7.0 ± 0.1 and 7.2 ± 0.1 for pretreatment with and without insulin, P <0.01). Almost similar results were found for KCl-induced contraction, both quantitatively and qualitatively. However, no shift in sensitivity (EC_{50}) after pretreatment with insulin was observed for KCl (Figure 3.4).

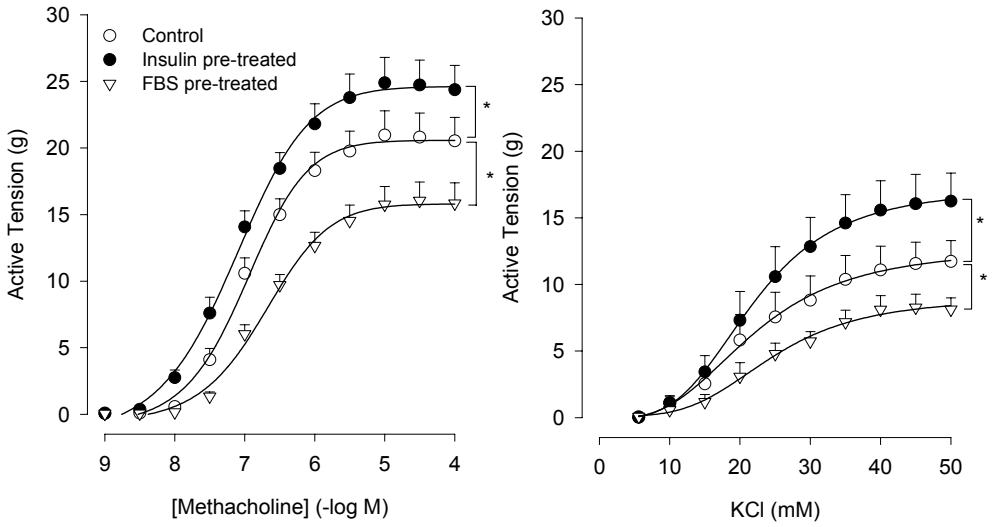

Figure 3.4 *Methacholine-(left panel) and KCl (right panel)-induced contraction of BTSM strips, pre-treated with serum-free medium (control), medium containg 10 % FBS or medium containing insulin (1 µM) for a period of 8 days. Data represent means ± s.e.mean of 8 experiments each performed in duplicate.*
** P<0.05.*

Discussion

As shown in this study, the acute effects of insulin on BTSM cells are dependent on the presence of other growth factors. Insulin, applied in a concentration generally used in ASM cell culture media was mitogenic by itself and augmented the proliferative effects induced by submaximally effective concentrations of PDGF, IGF-1 and EGF [12]. This augmentation was more profound for PDGF and EGF when compared to IGF-1, which is in line with results obtained by others using human ASM cells [6]. Probably structural similarities between insulin and IGF-1 cause these two growth factors to act to some extent through the same receptors [13].

In contrast to the acute effects of insulin, *pretreatment* with insulin induced a decrease in proliferative responsiveness. The presence of insulin during the quiescence period may have stimulated the cells to proliferate to a small extent, resulting in fewer cells that are available for stimulation by other growth factors. However, if this were the explanation for the decreased proliferative responses seen after pretreatment with insulin, basal thymidine incorporation should have been decreased as well. Moreover, proliferative responses to all growth factors should have been equally decreased. However, basal incorporated activity was similar for

control and insulin pretreated cells, demonstrating that insulin-induced differences occurred selectively at the level of growth factor-induced thymidine incorporation. Furthermore, the decrease in proliferative responsiveness was dependent on the growth factor applied: the PDGF response was diminished by approximately 50 %, whereas the EGF response was abolished. Since insulin pretreatment also increased contractility, the results indicate that insulin *pretreatment* induced a phenotypic shift towards a hypercontractile and less proliferative phenotype.

One could argue that the smooth muscle cells in strip preparations maintained in insulin are simply more viable due to the very presence of insulin and therefore respond more efficiently after 8 days in organ culture. However, serum-free maintained strips exhibit increased rather than decreased contractile responses as compared to freshly isolated strips. Moreover, growth factors which would stimulate rather than inhibit the number of viable cells, decrease contractility of BTSM strips as demonstrated in Chapter 2.

It is important to note that the increase in contractility after pretreatment with insulin and the decreased contractility after treatment with FBS and other growth factors are observed for both methacholine and KCl-induced contraction. Methacholine requires receptor-induced stimulation of phosphoinositide turnover to induce calcium release, whereas KCl uses voltage dependent calcium channels to induce calcium influx [14]. Therefore, qualitative and quantitative similarities between KCl and methacholine-induced contraction can be achieved only by modulating contraction downstream of intracellular Ca^{2+}-increases. Considering the long-term nature of the change in contractility (c.f. Chapter 2), changes at the level of the contractile machinery are the most likely explanation for the observed effects.

The hypercontractile phenotype is somewhat unexpected, since the results in Chapter 2 show that regulation of contractility by growth factors, including IGF-1, is reciprocally related to their mitogenic responses. Differences in the balance of activation of distinct kinase-isoforms may underlie this discrepancy: *e.g.* Akt1 and Akt2 are known to have opposite effects on skeletal muscle differentiation induced by insulin [15]. These kinases both act downstream of phosphoinositide 3- kinase (PI 3-kinase). It should be noted that PI 3-kinase is involved, at least in part, in the growth factor-induced phenotype shift (Chapter 2).

Previous studies concerning a role for insulin in ASM phenotype switching are not available. However, insulin is often used as a substituent in serum-free media, in which others have succeeded in inducing a hypercontractile canine ASM phenotype [3]. However, this was attributed to serum deprivation rather than to the presence of insulin [16]. Indeed, in chick gizzard smooth muscle cells, insulin has been shown to be involved in phenotypic switching to a hypercontractile phenotype [17]. In addition, prolonged treatment of PAC1 cells with insulin induces a switch from a vascular smooth muscle phenotype to a skeletal muscle phenotype as demonstrated by the expression of skeletal muscle specific proteins. Interestingly, RT-PCR analysis in these cells showed that this smooth muscle to skeletal muscle differentiation is

accompanied by increases in smooth muscle specific protein expression, such as myosin-light chain kinase (sm-MLCK), smooth muscle heavy chain (sm-MHC) and sm-calponin [18]. These findings suggest that insulin-induced changes toward a (hyper)contractile phenotype may not be confined to smooth muscle of bovine tracheal origin.

A lower prevalence of asthma and atopy symptoms in patients with type I diabetes mellitus has been reported in a number of epidemiological studies [11]; [10;19], although this is also debated [20]. The mechanism of this putative association is still unclear. Based on the present study, low plasma levels of insulin might be protective towards symptoms of asthma, since insulin may extend the range of airway smooth muscle phenotypic shifting either toward a proliferative or a hypercontractile phenotype, conditional on the presence of other growth factors. This could also contribute to the controversy with respect to the negative association of asthma and diabetes mellitus, since diabetics that are under well-controlled insulin treatment would be equally subjective to asthma as non-diabetic individuals. In line with this hypothesis, an increased function of inhibitory prejunctional muscarinic M_2-receptors and a decreased antigen challenge-induced influx of inflammatory cells in the airways have been demonstrated in rat model of streptozotocin-induced type I diabetes which could be reversed by the administration of insulin [21;22]. Using the same model, a diminished tracheal contractility was observed in long-term (8 week) diabetic rats [23], but not in 1 week diabetic rats [21;24]. A similar time-dependency has been observed for calmodulin expression [25]. Phenotype switching *in vivo* may be a slower process than *in vitro*, since it is still continuing 35 days after the last challenge in repeatedly allergen-challenged rats [26], whereas growth factor induced phenotype switching in intact BTSM *in vitro* is characterized by a t½ of 2.8 days (Chapter 2).

The long-term effects of insulin on ASM phenotype switching may also be important in view of recent human studies on the effectiveness of aerosolized insulin in diabetes management [7-9]. If used for diabetes treatment, lung concentrations of insulin will be chronically elevated as compared to other ways of administration. In diabetics suffering from airway diseases such as asthma as well, such treatment may worsen ASM hyperplasia and contractility by extending the phenotype switching capacity.

In conclusion, insulin is mitogenic and potentiates mitogenesis induced by other growth factors. In contrast, *pre*-treatment with insulin induces a hypercontractile and hypoproliferative BTSM phenotype. Therefore, insulin may enhance either contractility or proliferation of ASM, dependent on the duration of exposure to insulin. This may provide an explanation for the putative negative association between asthma and type I diabetes. In addition, this shows that aerosolized administration of insulin may result in adverse effects on airway smooth muscle mass and function.

Acknowlegdement
This study was financially supported by the Netherlands Asthma Foundation, grant NAF 99.53.

References
1. Halayko AJ and Solway J, Molecular mechanisms of phenotypic plasticity in smooth muscle cells. J.Appl.Physiol 90: 358-368, 2001.
2. Halayko AJ, Salari H, MA X, and Stephens NL, Markers of airway smooth muscle cell phenotype. Am.J.Physiol 270: L1040-L1051, 1996.
3. Ma X, Wang Y, and Stephens NL. Serum deprivation induces a unique hypercontractile phenotype of cultured smooth muscle cells. Am.J.Physiol. 274: C1206-C1214, 1998.
4. Hirst SJ, Twort CH, and Lee TH, Differential effects of extracellular matrix proteins on human airway smooth muscle cell proliferation and phenotype. Am.J.Respir.Cell Mol.Biol. 23: 335-344, 2000.
5. Gosens R, Meurs H, Bromhaar MM, McKay S, Nelemans SA, and Zaagsma J, Functional characterization of serum- and growth factor-induced phenotypic changes in intact bovine tracheal smooth muscle. Br.J.Pharmacol. 137: 459-466, 2002.
6. Ediger TL and Toews ML. Synergistic stimulation of airway smooth muscle cell mitogenesis. J.Pharmacol.Exp.Ther. 294:1076-1082, 2000.
7. Steiner S, Pfutzner A, Wilson BR, Harzer O, Heinemann L, and Rave K, Technosphere/Insulin--proof of concept study with a new insulin formulation for pulmonary delivery. Exp.Clin.Endocrinol.Diabetes 110: 17-21, 2002.
8. Henry RR, Mudaliar SR, Howland III WC, Chu N, Kim D, An B, and Reinhardt RR, Inhaled Insulin Using the AERx Insulin Diabetes Management System in Healthy and Asthmatic Subjects. Diabetes Care 26: 764-769, 2003.
9. Skyler JS, Cefalu WT, Kourides IA, Landschulz WH, Balagtas CC, Cheng SL, and Gelfand RA, Efficacy of inhaled human insulin in type 1 diabetes mellitus: a randomised proof-of-concept study. Lancet 357: 331-335, 2001.
10. Douek IF, Leech NJ, Gillmor HA, Bingley PJ, and Gale EA, Children with type-1 diabetes and their unaffected siblings have fewer symptoms of asthma. Lancet 353: 1850, 1999.
11. Abrahamson EM. Asthma, diabetes mellitus and hyperinsulinism. J.Clin.Endocrinol. 1, 402-406. 1941.
12. Kelleher MD, Abe MK, Chao TSO, Jain M, Green JM, Solway J, Rosner MR, and Hershenson MB. Role of MAP kinase activation in bovine tracheal smooth muscle mitogenesis. The-Am.J.Physiol. 268: L894-901,1995.
13. Bayes-Genis A, Conover CA, and Schwartz RS, The insulin-like growth factor axis: A review of atherosclerosis and restenosis. Circ.Res. 86: 125-130, 2000.
14. Zaagsma J, Roffel AF, and Meurs H, Muscarinic control of airway function. Life Sci. 60: 1061-1068, 1997.
15. Sumitani S, Goya K, Testa JR, Kouhara H, and Kasayama S, Akt1 and Akt2 differently regulate muscle creatine kinase and myogenin gene transcription in insulin-induced differentiation of C2C12 myoblasts. Endocrinology 143: 820-828, 2002.
16. Halayko AJ, Camoretti-Mercado B, Forsythe SM, Vieira JE, Mitchell RW, Wylam ME, Hershenson MB, and Solway J. Divergent differentiation paths in airway smooth muscle culture: Induction of functionally contractile myocytes. Am.J.Physiol. 276: L197-L206, 1999.

17. Hayashi K, Saga H, Chimori Y, Kimura K, Yamanaka Y, and Sobue K, Differentiated phenotype of smooth muscle cells depends on signaling pathways through insulin-like growth factors and phosphatidylinositol 3- kinase. J.Biol.Chem. 273: 28860-28867, 1998.
18. Graves DC and Yablonka-Reuveni Z, Vascular smooth muscle cells spontaneously adopt a skeletal muscle phenotype: a unique Myf5(-)/MyoD(+) myogenic program. J.Histochem.Cytochem. 48: 1173-1193, 2000.
19. Meerwaldt R, Odink RJ, Landaeta R, Aarts F, Brunekreef B, Gerritsen J, Van Aalderen WM, and Hoekstra MO, A lower prevalence of atopy symptoms in children with type 1 diabetes mellitus. Clin.Exp.Allergy 32: 254-255, 2002.
20. Stromberg LG, Ludvigsson GJ, and Bjorksten B, Atopic allergy and delayed hypersensitivity in children with diabetes. J.Allergy Clin.Immunol. 96: 188-192, 1995.
21. Coulson FR, Jacoby DB, and Fryer AD, Increased function of inhibitory neuronal M2 muscarinic receptors in trachea and ileum of diabetic rats. Br.J.Pharmacol. 135: 1355-1362, 2002.
22. Belmonte KE, Fryer AD, and Costello RW, Role of insulin in antigen-induced airway eosinophilia and neuronal M2 muscarinic receptor dysfunction. J.Appl.Physiol 85: 1708-1718, 1998.
23. Cros G, Gies JP, Cahard D, Cohen P, Filipek B, Mongold JJ, and Serrano JJ, Impairment of contractility associated with muscarinic supersensitivity in trachea isolated from diabetic rats: lack of correlation with ultrastructural changes or quinuclidinyl benzylate binding to lung membranes. Mol.Cell Biochem. 109: 181-183, 1992.
24. Belmonte KE, Jacoby DB, and Fryer AD, Increased function of inhibitory neuronal M2 muscarinic receptors in diabetic rat lungs. Br.J.Pharmacol. 121: 1287-1294, 1997.
25. Ozturk Y, Aydin S, Altan VM, Yildizoglu-Ari N, and Ozcelikay AT, Effect of short and long term streptozotocin diabetes on smooth muscle calmodulin levels in the rat. Cell Calcium 16: 81-86, 1994.
26. Moir LM, Leung SY, Eynott PR, McVicker CG, Ward JP, Chung KF, and Hirst SJ, Repeated allergen inhalation induces phenotypic modulation of smooth muscle in bronchioles of sensitized rats. Am.J.Physiol. 284: L148-L159, 2003.